Published in final edited form as:

*Nature*. 2017 March 16; 543(7645): 438–442. doi:10.1038/nature21423.

# KICSTOR recruits GATOR1 to the lysosome and is necessary for nutrients to regulate mTORC1

**Rachel L. Wolfson**[1,2,3,4,*], **Lynne Chantranupong**[1,2,3,4,*], **Gregory A. Wyant**[1,2,3,4], **Xin Gu**[1,2,3,4], **Jose M. Orozco**[1,2,3,4], **Kuang Shen**[1,2,3,4], **Kendall J. Condon**[1,2,3,4], **Sabrina Petri**[5], **Jibril Kedir**[1,2,3,4], **Sonia M. Scaria**[1,2,3,4], **Monther Abu-Remaileh**[1,2,3,4], **Wayne N. Frankel**[5], and **David M. Sabatini**[1,2,3,4]

[1]Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Department of Biology, 9 Cambridge Center, Cambridge, MA 02142, USA

[2]Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[3]Koch Institute for Integrative Cancer Research, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

[4]Broad Institute of Harvard and Massachusetts Institute of Technology, 7 Cambridge Center, Cambridge MA 02142, USA

[5]Department of Genetics and Development and Institute for Genomic Medicine, Columbia University Medical Center, New York, NY 10032, USA

## Abstract

The mechanistic target of rapamycin complex 1 kinase (mTORC1) is a central regulator of cell growth that responds to diverse environmental signals and is deregulated in many human diseases, including cancer and epilepsy[1–3]. Amino acids are a key input, and act through the Rag GTPases to promote the translocation of mTORC1 to the lysosomal surface, its site of activation[4]. Multiple protein complexes regulate the Rag GTPases in response to amino acids, including GATOR1, a GTPase activating protein for RagA, and GATOR2, a positive regulator of unknown molecular function. Here, we identify a four-membered protein complex (KICSTOR) composed of the KPTN, ITFG2, C12orf66, and SZT2 gene products as required for amino acid or glucose



Correspondence should be addressed to D.M.S. Tel: 617-258-6407; Fax: 617-452-3566; sabatini@wi.mit.edu.

*These authors contributed equally to this work

**Author Contributions**

R.L.W., L.C., and D.M.S. formulated the research plan and interpreted experimental results. R.L.W. and L.C. designed and performed most experiments with assistance from G.A.W., X.G., J.M.O, K.S., K.J.C., J.K., S.M.S., and M.A.R. X.G. undertook the epistasis experiments, G.A.W. those using purified lysosomes, J.M.O. those involving glucose signaling, and K.S. those using size exclusion chromatography. S.P. and W.N.F. generated the *Szt2* gene trap mice and harvested tissues, which were analyzed by R.L.W. and G.A.W. R.L.W. and D.M.S. wrote the manuscript and all authors edited it.

**Competing Financial Interests**

D.M.S. is a founder, consultant, and shareholder of Navitor Pharmaceuticals, Inc., which is targeting the amino acid sensing pathway for therapeutic benefit. R.L.W., L.C., and J.M.O. are shareholders of Navitor Pharmaceuticals. R.L.W., L.C., J.M.O., D.M.S., and the Whitehead Institute have filed two provisional patents that relate to amino acid sensing by the mTOR pathway.

deprivation to inhibit mTORC1 in cultured cells. In mice lacking SZT2, mTORC1 signaling is increased in several tissues, including in neurons in the brain. KICSTOR localizes to lysosomes; binds to GATOR1 and recruits it, but not GATOR2, to the lysosomal surface; and is necessary for the interaction of GATOR1 with its substrates, the Rag GTPases, and with GATOR2. Interestingly, several KICSTOR components are mutated in neurological diseases associated with mutations that lead to hyperactive mTORC1 signaling[5–10]. Thus, KICSTOR is a lysosome-associated negative regulator of mTORC1 signaling that, like GATOR1, is mutated in human disease[11,12].

---

To search for GATOR1-interacting proteins that may have escaped prior identification, we used the CRISPR/Cas9 system to engineer the *DEPDC5* gene in HEK-293T cells to express a FLAG-tagged version of DEPDC5, a GATOR1 component, at endogenous levels. Mass spectrometric analysis of FLAG-immunoprecipitates prepared from these cells revealed the presence of GATOR2, as well as four proteins of unknown function encoded by the *KPTN*, *ITFG2*, *C12orf66*, and *SZT2* genes and of predicted molecular weights of 48, 49, 50, and 380 kDa, respectively (Fig. 1a). As shown below, these proteins form a complex, which we named KICSTOR for KPTN, ITFG2, C12orf66, and SZT2-containing regulator of mTORC1. KICSTOR components are conserved in vertebrates but not fungi (Fig. 1b). Some non-vertebrates, like *Caenorhabditis elegans*, encode homologues of *SZT2* but not of *KPTN*, *ITFG2*, or *C12orf66*, while others, including *Drosophila melanogaster*, lack all four KICSTOR components (Fig. 1b).

Endogenous KICSTOR was present in anti-FLAG immunoprecipitates prepared from cells expressing endogenously FLAG-tagged DEPDC5 or WDR59, a GATOR2 component (Extended Data Fig. 1a, b). Importantly, an anti-KPTN antibody co-immunoprecipitated SZT2, ITFG2, and C12orf66, as well as GATOR1 and GATOR2 components (Extended Data Fig. 1c). Amino acid starvation and stimulation of cells did not significantly affect the interactions between any complex components (Extended Data Fig. 1a, b, c). Thus, GATOR1 and GATOR2 associate with KICSTOR in an amino acid insensitive fashion.

Using HEK-293T cells transiently expressing recombinant versions of the KICSTOR components, we explored how they interact with each other. KPTN and ITFG2 associated even in the absence of the co-expression of either C12orf66 or SZT2 (Extended Data Fig. 1d), suggesting that they form a heterodimer on their own. Indeed, KPTN and ITFG2 associated in cells deficient for SZT2 or C12orf66, and loss of ITFG2 severely reduced KPTN levels and vice versa (Extended Data Fig. 1e, f, g). The KPTN-ITFG2 heterodimer co-immunoprecipitated C12orf66 only when SZT2 was also expressed and did not compete with C12orf66 for interacting with SZT2 (Extended Data Fig. 1d, h). In size-exclusion chromatography analyses of cell lysates, C12orf66, KPTN, and ITFG2 fractionated into two main pools, one that co-migrated at a very high molecular weight with SZT2 and was absent in SZT2-deficient cells, and a more abundant pool that migrated at lower molecular weights (Extended Data Fig. 2). Collectively, these results suggest that the four KICSTOR proteins form a large complex in which SZT2 serves as the link between the other three.

We readily detected endogenous KICSTOR in anti-FLAG immunoprecipitates from HEK-293E cells stably expressing FLAG-tagged Nprl2, a GATOR1 component, but FLAG-tagged WDR24, a GATOR2 component, co-immunoprecipitated much lower amounts of

KICSTOR (Fig. 1c). Consistent with the GATOR2-KICSTOR association being indirect, loss of GATOR1 strongly reduced it (Fig. 1d), while that of GATOR2 did not significantly affect the GATOR1-KICSTOR interaction (Fig. 1e). SZT2 co-immunoprecipitated with GATOR1 even in cells lacking other KICSTOR components (Extended Data Fig. 3a) and was required for GATOR1 or GATOR2 to co-immunoprecipitate the KPTN-ITFG2 heterodimer (Extended Data Fig. 3b). Moreover, KPTN-ITFG2, C12orf66, and GATOR1 co-immunoprecipitated three fragments of the 380 kDa SZT2 to different extents and the integrity of KICSTOR did not depend on either GATOR complex (Extended Data Fig. 3c, d, e, f). Altogether, these results suggest that KICSTOR is a distinct four-protein complex that uses its SZT2 component to interact with GATOR1, which in turn binds to GATOR2 (Fig. 1f). Consistent with this model, the very high molecular weight pool of Nprl3 detected by size-exclusion chromatography was absent in SZT2-deficient cells (Extended Data Fig. 2).

Because much of the nutrient sensing machinery upstream of mTORC1, including the Rag GTPases, localizes to the lysosomal surface[4,13], we asked if KICSTOR is also there. Indeed, examination of HeLa cells expressing GFP-tagged ITFG2 (as a marker of KICSTOR) at levels 2–3 fold above the endogenous protein, revealed that KICSTOR localizes, at least in part, to lysosomes in an amino acid-insensitive fashion (Fig. 1g, and Extended Data Fig. 4a, b). The GATOR complexes have been reported to localize to the lysosome[14–17], which we confirmed in HeLa cells stably expressing GFP-tagged components of GATOR1 (Nprl2) or GATOR2 (Mios and WDR24) (Fig. 1h, i and Extended Data Fig. 4a, b, c). Consistent with these results, amino acids did not affect the amounts of endogenous GATOR1, GATOR2, or KICSTOR present on immunopurified lysosomes, but, as expected, did regulate that of mTORC1 (Extended Data Fig. 4d). The lysosomes were not contaminated with mitochondria, Golgi, ER, or peroxisomes (Extended Data Fig. 4d).

Given the strong interaction between KICSTOR and GATOR1, an inhibitor of the Rag GTPases, we reasoned that KICSTOR might be required for the control of the mTORC1 pathway by amino acids. Indeed, in HEK-293T cells lacking any KICSTOR subunit, amino acid deprivation did not inhibit mTORC1 signaling, as detected by the phosphorylation of S6K1, 4E-BP1, and ULK1, or induce autophagy (Fig. 2a, b and Extended Data Fig. 1e and 5a, b, c). In HeLa cells loss of either SZT2 or ITFG2 had similar effects on S6K1 phosphorylation (Extended Data Fig. 5d, e, f). Although less is known about how glucose is sensed upstream of the Rag GTPases[18,19], loss of any KICSTOR component in HEK-293T cells also rendered mTORC1 signaling insensitive to glucose starvation (Fig. 2c). Glucose did not affect the lysosomal localization of GATOR1 and serum deprivation and insulin stimulation still regulated mTORC1 in HeLa cells lacking KICSTOR components (Extended Data Fig. 5g, h, i, j).

To determine if KICSTOR also inhibits mTORC1 signaling *in vivo* we analyzed previously generated mice in which the *Szt2* gene was disrupted by a gene trap ($Szt2^{GT/GT}$)[20]. Compared to wild-type littermates, mTORC1 signaling was increased in the liver and skeletal muscle of fasted *Szt2* gene trap mice as assessed by the phosphorylation of S6, a substrate of S6K1, and of 4E-BP1 (Fig. 2d, e and Extended Data Fig. 6a, b). Immunohistochemical detection of phospho-S6 in tissue slices from the brain as well as liver and heart revealed increases in mTORC1 signaling in cerebellar and cortical neurons and

hepatocytes and cardiomyocytes of the *Szt2*$^{GT/GT}$ mice (Fig. 2f and Extended Data Fig. 6c). Thus, loss of the SZT2 component of KICSTOR increases mTORC1 signaling in multiple mouse tissues *in vivo*.

To define where KICSTOR acts in the mTORC1 pathway, we performed epistasis experiments between KICSTOR and established components of the pathway. Expression of the dominant negative Rag GTPases (RagB$^{T54N}$-RagC$^{Q120L}$) still inhibited mTORC1 in SZT2-deficient cells, indicating that KICSTOR is upstream of the Rag GTPases (Fig. 3a). While great overexpression of GATOR1 suppressed mTORC1 signaling in both wild-type and SZT2-deficient cells (Fig. 3a), much lower levels of GATOR1 overexpression reduced mTORC1 signaling to a lesser extent in the SZT2-deficient than wild-type cells, suggesting that GATOR1 requires SZT2 to efficiently exert its inhibitory effects (Fig. 3b). Loss of GATOR2 strongly inhibited mTORC1 signaling in wild-type cells, but did not affect the constitutive signaling of cells deficient for SZT2 (Fig. 3c) or Nprl3 (Extended Data Fig. 7a). Thus, KICSTOR, likely acting in concert with GATOR1, functions downstream of or in parallel to GATOR2 to negatively regulate Rag GTPase signaling to mTORC1. Consistent with this interpretation, loss of KICSTOR, like that of GATOR1[17], was sufficient to drive mTORC1 to the lysosomal surface in cells starved of amino acids (Fig. 3d and Extended Data Fig. 7b).

While Ragulator tethers the Rags to the lysosomal surface[13], it is unknown what recruits GATOR1 there and so we considered such a role for KICSTOR. This was the case as in cells deficient for KICSTOR, GATOR1 no longer localized to lysosomes and instead was dispersed throughout the cytoplasm (Fig. 4a, b, and Extended Data Fig. 8a). In contrast, loss of KICSTOR had little effect on the lysosomal localization of GATOR2 or the Rag GTPases (Extended Data Fig. 8b, c). In agreement with the imaging results, loss of SZT2 very strongly decreased the amount of GATOR1, but not GATOR2, on purified lysosomes (Fig. 4c). Consistent with the amino acid-insensitive lysosomal localization of mTOR in cells deficient for SZT2 (Fig. 3d), amino acid starvation did not reduce the amount of mTORC1 on lysosomes isolated from these cells (Fig. 4c). Given the importance of KICSTOR for localizing GATOR1 to lysosomes, we reasoned that KICSTOR should be necessary for GATOR1 to interact with its substrates the Rag GTPases, as well as with GATOR2. Indeed, loss of SZT2 strongly reduced the amounts of RagA and RagC and GATOR2 that co-immunoprecipitated with GATOR1 (Fig. 4d and Extended Data Fig. 9a, b, c, d). As previously reported[17], amino acid stimulation reduced the interaction of GATOR1 with the Rag GTPases in wild-type cells (Fig. 4d). Thus, KICSTOR has at least two molecular functions that can explain its loss of function phenotype: KICSTOR targets GATOR1 to the lysosomal surface where its substrates, the Rag GTPases, and its potential regulator, GATOR2, reside (Fig. 4e). KICSTOR is a large complex so it likely has additional roles in mTORC1 signaling beyond those we have defined, but the protein sequences of its components do not suggest any biochemical functions. It was not possible to ascertain if tethering of GATOR1 to the lysosomal surface bypasses the need for KICSTOR for the control of mTORC1 signaling by nutrients because the addition to any GATOR1 component, on its N- or C-terminus, of the lysosomal targeting sequence of LAMTOR1 prevented formation of GATOR1 or its interaction with GATOR2.

It will be important to understand if our findings have therapeutic implications because mutations in *KPTN* and *SZT2* and loss of the genomic locus containing *C12orf66* have been identified in patients with epilepsy and brain malformation disorders[5–9]. The fact that the same diseases are associated with loss of function mutations in GATOR1[12] and activating mutations in mTOR[21], support the notion that KICSTOR is a negative regulator of the mTORC1 pathway. Consistent with the phenotypes of patients with mutations in KICSTOR components, the few mice deficient in *Szt2* that survive to adulthood are more susceptible to epileptic seizures[20]. If, as in mice, KICSTOR mutations in humans also activate neuronal mTORC1, patients with these mutations might benefit from inhibition of mTORC1 with drugs like rapamycin.

## Methods

### Materials

Reagents were obtained from the following sources: antibodies to LAMP2 (sc-18822), ITFG2 (SC 134686), and HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; the antibody to PEX19 (ab137072) from Abcam; the antibody to raptor from EMD Millipore (2818718); the antibody to Sec13 from Gene Tex (GTX 101055); antibodies to phospho-T389 S6K1 (9234), S6K1 (2708), phospho-S235/236 S6 (2211), S6 (2217), phospho-S65 4E-BP1 (9451), 4E-BP1 (9644), phospho-757 ULK1 (6888), ULK1 (8054), phospho-792-raptor (2083), phospho-79-ACC (3661), ACC (3662), phospho-T308-Akt (4056), Akt (4691), LC3B (2775), mTOR (2983), RagC (3360), Mios (13557), VDAC (4661), Calreticulin (12238), Golgin-97 (13192), Cathepsin D (2284), and the myc (2278) and FLAG (2368) epitopes from Cell Signaling Technology (CST); antibodies to the HA epitope from CST (3724) and Bethyl laboratories (A190208A); antibody to KPTN from ProteinTech (16094-1AP); antibody to Nprl3 from Sigma (HPA0011741). RPMI, FLAG M2 affinity gel, and amino acids from Sigma Aldrich; DMEM from SAFC Biosciences; XtremeGene9 and Complete Protease Cocktail from Roche; Alexa 488 and 568-conjugated secondary antibodies; Inactivated Fetal Bovine Serum (IFS) from Invitrogen; and amino acid-free RPMI from US Biologicals. Jianxin Xie (Cell Signaling Technology) generously provided the DEPDC5, Mios, Nprl2, WDR24, WDR59 (53385), C12orf66, Seh1L, and SZT2 antibodies. The C12orf66 and SZT2 antibodies are bleeds. At the beginning of this project we also used an antibody to SZT2 from Abcam (SZT2 blots in Fig. 1c and Extended Data Fig. 5d), but it has since been discontinued and is no longer available.

### Cell lines and tissue culture

HEK-293T, HEK-293E, and HeLa cells were cultured in DMEM 10% IFS (inactivated fetal bovine serum) supplemented with 2 mM glutamine. All cell lines were maintained at 37°C and 5% CO2. All cell lines were obtained from ATCC (American Type Culture Collection) and validated and tested for mycoplasma.

### Cell lysis, immunoprecipitations, transfections, and lysosomal purifications

Cells were rinsed once with ice-cold PBS and lysed immediately with Triton lysis buffer (1% Triton, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM Hepes pH 7.4, 2.5

mM $MgCl_2$ and 1 tablet of EDTA-free protease inhibitor [Roche] (per 25 ml buffer). The cell lysates were clarified by centrifugation at 13,000 rpm at 4°C in a microcentrifuge for 10 minutes. For anti-FLAG-immunoprecipitations, the FLAG-M2 affinity gel was washed with lysis buffer 3 times. 30 μl of a 50% slurry of the affinity gel was then added to cleared cell lysates and incubated with rotation for 2 hours at 4°C. The beads were washed 3 times with lysis buffer containing 500 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample buffer and boiling for 5 minutes as described[22], resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting.

For transfection experiments in HEK-293T cells, 2 million cells were plated in 10 cm culture dishes. Twenty-four hours later, cells were transfected via the polyethylenimine method[23] with the pRK5-based cDNA expression plasmids indicated in the figures. The total amount of plasmid DNA in each transfection was normalized to 5 μg with empty pRK5. Thirty-six hours after transfection, cells were lysed as described above.

For experiments which required amino acid starvation or restimulation, cells were treated as previously described[24]. Briefly, cells were incubated in amino acid free RPMI for 50 minutes and then stimulated with amino acids for 10 minutes. The amino acid mixture used was previously described[17]. For glucose starvation, cells were incubated in RPMI media lacking glucose but containing amino acids and dialyzed serum for 50 minutes, followed by a 10-minute restimulation with 5 mM D-Glucose. For insulin stimulation experiments, cells were incubated in RPMI without serum for 50 minutes and restimulated with 1 μg/ml insulin for 10 minutes.

Lysosomes were purified via immunopurification from wild-type or engineered HEK-293T cells stably expressing HA-RFP-LAMP1 as previously described[25].

### Identification of KICSTOR by immunoprecipitation followed by mass spectrometry

Immunoprecipitates from HEK-293T cells expressing endogenously tagged 3xFLAG-DEPDC5 were prepared using Triton lysis buffer. Proteins were eluted with the FLAG peptide (sequence DYKDDDDK) from the FLAG-M2 affinity gel, resolved on 4–12% NuPage gels (Invitrogen), and stained with simply blue stain (Invitrogen). Each gel lane was sliced into 10–12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described[4]. Peptides corresponding to KICSTOR components were detected in the FLAG-DEPDC5 immunoprecipitates, while no peptides were detected in negative control immunoprecipitates of FLAG-metap2.

### Evolutionary and domain analysis of the KICSTOR components

To assess the conservation of the proteins in the KICSTOR complex, analysis was performed using the PHMMER software (https://www.ebi.ac.uk/Tools/hmmer/search/phmmer).

## Generation of cells with loss of function mutations in GATOR2, GATOR1, or KICSTOR components

To generate HEK-293T or HeLa cells with reduced expression of GATOR2, GATOR1, or KICSTOR components, sense (S) and antisense (AS) oligonucleotides encoding the following single guide RNAs (sgRNAs) were cloned into the pX330 vector. Cells treated with the sgRNA targeting the AAVS1 locus served as negative control cells.

sgAAVS1_S: caccgTCCCCTCCACCCCACAGTG

sgAAVS1_AS: aaacCACTGTGGGGTGGAGGGGAc

sgSZT2_1S: caccgGAAGCAGCCCGCCTAAGCAG

sgSTZ2_1AS: aaacGAAGCAGCCCGCCTAAGCAGc

sgSZT2_2S: caccgGTGGCAGCCAGATGAACCAG

sgSTZ2_2AS: aaacCTGGTTCATCTGGCTGCCACc

sgSZT2_3S: caccgAACACGGGTGGAAGTGACGA

sgSTZ2_3AS: aaacTCGTCACTTCCACCCGTGTTc

sgWDR24_1S: caccgACCCAGGGCTGTGGTCACAC

sgWDR24_1AS: aaacTCAGGAGTACTCGCAGAGGTc

sgNprl3_1S: caccGGCTTTCAGGCTCCGTTCGA

sgNprl3_1AS: aaacTCGAACGGAGCCTGAAAGCC

sgKPTN_1S: caccgATCACATCAGTAAACATGAG

sgKPTN_1AS: aaacCTCATGTTTACTGATGTGATc

sgITFG2_1S: caccgACCCAGGGCTGTGGTCACAC

sgITFG2_1AS: aaacGTGTGACCACAGCCCTGGGTc

sgC12orf66_3S: caccgGGCTAAGGACAATGTGGAGA

sgC12orf66_3AS: aaacTCTCCACATTGTCCTTAGCCc

On day one, 200,000 HEK-293T cells were seeded into 6 wells of a 6-well plate. Twenty-four hours post seeding, each well was transfected with 250 ng shGFP pLKO, 1 ug of the pX330 guide construct, 0.5 ug of empty pRK5 using XtremeGene9. The following day, cells were trypsinized, pooled in a 10 cm dish, and selected with puromycin to eliminate untransfected cells. Forty-eight hours after selection, the media was aspirated and replenished with fresh media lacking puromycin. The following day, cells were single cell sorted with a flow cytometer into the wells of a 96-well plate containing 150 ul of DMEM supplemented with 30% IFS.

For HeLa cells, on day one 1 million cells were plated into a 10 cm dish. Twenty-four hours later, the cells were transfected with 1 ug pLJM1 GFP and 300 ng of the pX330 guide construct using XtremeGene9. Selection with puromycin was started the following day to eliminate untransfected cells. Forty-eight hours after selection, the media was aspirated and

replenished with fresh media lacking puromycin and the cells were single cell sorted as described above. Cells were grown for two weeks and the resultant colonies were trypsinized and expanded. Cell clones were validated for reduced expression of the relevant proteins via immunoblotting (SZT2, KPTN, ITFG2, Nprl3, WDR24) or by confirming that the targeted exon contains out of frame mutations via genomic DNA sequencing (*C12orf66*). Most of the cell clones we generated likely do not express the targeted proteins, but because there is the possibility that some expression remains below the limit of detection of the available antibodies, we do not label the clones as null. We suspect that the cell clone generated with the sgRNA targeting the *WDR24* gene expresses a very low amount of the WDR24 protein that is below the limit of detection of the available antibody.

To generate HEK-293T or HeLa cells stably expressing the indicated sgRNAs, the following oligonucleotides were cloned into the pLenti viral vectors:

sgAAVS1_S: caccgTCCCCTCCACCCCACAGTG

sgAAVS1_AS: aaacCACTGTGGGGTGGAGGGGAc

sgKPTN_1S: caccgGCGCAACGGACAAGGCCCCG

sgKPTN_1AS: aaacCGGGGCCTTGTCCGTTGCGCc

sgKPTN_2S: caccgGCAGAGCAATGTGTACGGGC

sgKPTN_2AS: aaacGCCCGTACACATTGCTCTGCc

sgKPTN_3S: caccgGAGCACCTTGCCTTTAAGGG

sgKPTN_3AS: aaacCCCTTAAAGGCAAGGTGCTCc

sgKPTN_6S: caccgGTCAAGGTTGTACTCAGAGC

sgKPTN_6AS: aaacGCTCTGAGTACAACCTTGACc

sgITFG2_1S: caccgGGTGGGAGACACCAGCGGGA

sgITFG2_1AS: aaacTCCCGCTGGTGTCTCCCACCc

sgITFG2_2S: caccgGAAGTTAAATGAACTGGTGG

sgITFG2_2AS: aaacCCACCAGTTCATTTAACTTCc

sgITFG2_3S: caccgAAAATGATGACAGTCGGCCA

sgITFG2_3AS: aaacTGGCCGACTGTCATCATTTTc

sgC12orf66_1S: caccgCGAGAGGCCAACAAGAGCGC

sgC12orf66_1AS: aaacGCGCTCTTGTTGGCCTCTCGc

sgC12orf66_3S: caccgGGCTAAGGACAATGTGGAGA

sgC12orf66_3AS: aaacTCTCCACATTGTCCTTAGCCc

To generate the lentiviruses, on day one 750,000 HEK-293T cells were seeded in a 6 well plate in DMEM supplemented with 20% inactivated fetal bovine serum (IFS). Twenty-fours hours later, the cells were transfected with the above sgRNA pLenti encoding plasmids

alongside the Delta VPR envelope and CMV VSV-G packaging plasmids using XtremeGene9 transfection reagent. Twelve hours post transfection, the spent media was aspirated and replaced with 2 ml fresh media. Virus-containing supernatants were collected 36 hours after replacing media and passed through a 0.45 micron filter to eliminate cells. One million HEK-293T or HeLa cells in the presence of 8 μg/ml polybrene (Millipore) were infected with 250 μl of virus for each construct in 1 ml total volume of media and then spun at 2,200 rpm for 45 minutes at 37°C. Forty-eight hours after selection, cells were trypsinized and selected with 1 μg/ml puromycin and seeded on the $10^{th}$ day for signaling experiments, as described. Cell lines were validated for reduced expression of the relevant proteins via immunoblotting (KPTN, ITFG2) or by confirming that the targeted exon contains out of frame mutations via genomic DNA sequencing (*C12orf66*).

### Generation of cells expressing endogenously FLAG-tagged WDR59 or DEPDC5

To insert an N-terminal 3xFLAG tag into the *WDR59* or *DEPDC5* genes, 200,000 HEK-293T cells were seeded into 6 wells of a 6 well plate. Twenty-four hours later, each well was transfected with the following constructs: 250 ng shGFP pLKO (to provide transient puromycin resistance), 1000 ng of the indicated single guide RNA (sgRNA), 500 ng of the indicated single stranded DNA oligos, and 5.25 ul XtremeGene9 transfection reagent.

Single stranded DNA oligos used for homologous recombination:

WDR59:

CTAGCTCACCTGGGAGTCACGGAACTCTACAACCACGTTTTCGCTGCTCCA
TCTTGCAGCGCCTGCGGCCGCCTTGTCATCGTCATCCTTGTAATCAATGTCA
TGATCTTTATAATCACCGTCATGGTCTTTGTAGTCCATCTCCCCCGCCCGGC
CGCCGCGGCCCCAGGACGGCGCCCTCCCACCCCGCCGTCCCCAGT

DEPDC5:

GGAGGCAAGATGACTTCTCTGCCCCAAGCTTGGAACAGCTAAAGGGAAAA
ACAGTGCAAGATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGA
CATTGATTACAAGGATGACGATGACAAGGCGGCCGCAGGCCGTACGACGA
AAGTCTACAAACTCGTCATCCACAAGAAGGGCTTTGGGGGCAGTGGTCA

sgRNAs cloned into pX330:

WDR59 sense: caccgCGGGGAGATGGCGGCGCGA

WDR59 antisense: aaacTCGCGCCGCCATGTCCCCCGc

DEPDC5 sense: caccgTGCAAGATGAGAACAACAA

DEPDC5 antisense: aaacTTGTTGTTCTCATCTTGCAc

Forty-eight hours after transfection, the media was removed from cells and replaced with fresh media supplemented with 2 ug/ml of puromycin. The following day, cells were trypsinized, pooled, and replated into 10 cm plates in media containing puromycin. Twenty-four hours later, the cell media was changed to fresh media lacking puromycin. Forty-eight hours later, cells were single cell sorted into 96 well plates in 150 ul of cell media.

Following two weeks of growth, individual clones were expanded. To identify clones containing a 3xFLAG tag incorporated into the endogenous gene, genomic DNA was extracted from each clone using QuickExtract DNA solution (Epicentre) as described[26]. The primers indicated below were used to amplify the genomic region surrounding the insertion site, PCR products were subcloned into pRK5, and plasmids were subsequently submitted for sequencing. Validated clones were tested for their response to amino acid starvation and stimulation compared to wild type HEK-293T cells to verify that incorporation of the epitope tag did not alter mTORC1 signaling.

Genomic PCR primers:

WDR59_F: TCCACTCGGCCTCTAGCTCA

WDR59_R: GAGGGCGTGCCTGTTTGTTG

DEPDC5_F: TTCCGAGAGTCACTTGGCAC

DEPDC5_R: AGTCGCCTGTTTAGCCTCAAT

### Generation of cells stably expressing cDNAs

For the GFP-tagged cDNAs, the indicated GATOR1, GATOR2, and KICSTOR components were cloned into the N-terminal GFP retroviral vector pIC242 or the C-terminal GFP retroviral vector KG371 in the case of Mios**.** For retrovirus production, 750,000 HEK-293T cells were seeded on Day 1 into a 6 well plate and were transfected 18–24 hours later with 1 ug of pIC242 or KG371 construct, gag/pol and the CMV VSV-G packaging plasmids using the XtremeGene9 transfection reagent. Twelve hours later the media was changed to fresh DMEM with 20% IFS, followed by collection of the virus 36 hours after transfection. The virus was filtered through a 0.45 um filter and added, with polybrene, to the appropriate HeLa cells in a 6 well plate (500,000 cells per well). Twelve hours later the media was changed to fresh media. Finally, twenty-four hours after infection the cells were trypsinized and plated into a 10 cm plate in the presence of 1 ug/ul blasticidin for three days. The following day, low expressing GFP cells were single cell sorted with a flow cytometer into the wells of a 96-well plate containing 150 ul of DMEM supplemented with 30% IFS. Cells were grown for two weeks and the resultant colonies were trypsinized and expanded. Clones were validated for expression of the relevant protein via immunoblotting. To generate HeLa cells deficient in KICSTOR and expressing GFP-tagged GATOR1 or GATOR2 components, the protocol for CRISPR/Cas9 modification of HeLa cells described previously was used, and the cells were subjected to a second round of cell sorting.

The lentiviral expression plasmids used were: pLJM1-FLAG-metap2, pLJM1-FLAG-WDR24, pLJM1-FLAG-Nprl2, and pLJC5-HA-Nprl3 for the interaction experiments and pLJM1-HA-RFP-LAMP1 for the lysosomal immunopurifications. Lentiviruses were produced by transfection of HEK-293T cells with the above plasmids in combination with VSV-G envelope and CMV ΔVPR 8 packaging plasmids. Twenty-four hours after transfection, the media was changed to DMEM with 20% IFS. Forty-eight hours after transfection, the virus-containing supernatant was collected from the cells and passed through a 0.45 μm filter. Target cells were plated in 6-well plates containing DMEM 10%

IFS with 8 μg/mL polybrene and infected with virus containing media. Twenty-four hours later, the media was changed to fresh media containing puromycin for selection.

### Immunofluorescence assays

Immunofluorescence assays were performed as described previously[13]. Briefly, 300,000 HEK-293T or 150,000 HeLa cells were plated on fibronectin-coated glass coverslips in 6-well tissue culture plates. Twenty-four hours later, the slides were rinsed with PBS once and fixed for 15 minutes with 4% paraformaldehyde in PBS at room temperature. The slides were rinsed three times with PBS and cells were permeabilized with 0.05% Triton X-100 in PBS for 5 minutes. After rinsing three times with PBS, the slides were blocked for 1 hour in Odyssey blocking buffer, and then incubated with primary antibody in Odyssey blocking buffer for 1 hour at room temperature, rinsed three times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:1000 in Odyssey blocking buffer) for 45 minutes at room temperature in the dark and washed three times with PBS. The primary antibodies used were directed against GFP (Rockland Immunochemicals; 1:500 dilution), LAMP2 (Santa Cruz Biotechnology; 1:300 dilution), mTOR (CST; 1:100-1:300 dilution), and RagC (CST; 1:100 dilution). Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) containing DAPI.

Images were acquired on a Zeiss AxioVert200M microscope with a 63× oil immersion objective and a Yokogawa CSU-22 spinning disk confocal head with a Borealis modification (Spectral Applied Research/Andor) and a Hamamatsu Orca-ER CCD camera. The MetaMorph software package (Molecular Devices) was used to control the hardware and image acquisition. Images were captured in the Cy3, Cy5, and DAPI channels. Although the DAPI channel is not shown in the main images, it is in the insets as a blue signal.

For quantitative analyses, the raw image files were opened in the Fiji software package[27] and a maximum intensity projection of a z-stack of ~6–8 contiguous focal planes (~0.5 μm each) was used. In each cell analyzed, a cytoplasmic region of interest containing lysosomes (high LAMP2 signal) was chosen and in this area the mean fluorescence intensities (MFIs) of the Cy5 (LAMP2) and Cy3 channels (GFP, mTOR, or RagC) were measured. In the same cell an equivalently sized area in a region of the cytoplasm not containing lysosomes (low LAMP2 signal) was chosen and the MFIs of the Cy5 and Cy3 channels were also measured in this area. For each channel, the MFI of the non-lysosomal area was subtracted from that of the lysosomal area. The value obtained for the Cy3 channel was then divided by the analogous value for the Cy5 channel to give the lysosomal enrichment factor shown in the bar graphs in the figures. A lysosomal enrichment factor close to 1 indicates that the Cy3 signal was enriched in a region of the cell containing lysosomes over one that does not. A lysosomal enrichment factor closer to 0 indicates that the Cy3 signal was not enriched at the lysosomes, indicating no specific co-localization with the Cy5 signal. For each condition studied, images of at least 3 distinct fields were captured and within each 2–5 cells were analyzed as described above so that at least 10 cells were analyzed per condition. The mean lysosomal enrichment factor was calculated for each field analyzed, and the mean of the means of these fields is shown (bar graphs show mean ± standard error of the mean). In our experiments, the MFIs in the Cy3 and Cy5 channels were of similar magnitudes and so we

obtained equivalent results regarding lysosomal co-localization whether we subtracted or divided the MFIs of the two areas in calculating the lysosomal enrichment factors. Two-tailed *t* tests were used for comparisons between conditions. All comparisons were two-sided, and p-values < 0.05 were considered significant. Variance was similar across groups.

### Analysis of mTORC1 signaling in *Szt2*$^{GT/GT}$ mice

Previously described homozygous 129S1.129P2-*Szt2*$^{Gt(XH662)Byg}$/*Szt2*$^{Gt(XH662)Byg}$ (hereafter *Szt2*$^{GT/GT}$) and wild-type mice[20] of 1–5 months in age were fasted for 8 hours beginning at 7 am. A total of 8 mice were used across experiments – four wild-type females, three *Szt2*$^{GT/GT}$ females and one *Szt2*$^{GT/GT}$ male. Sample size was determined based on variation in mTORC1 signaling observed in wild-type mice. Mice were sacrificed and the liver, gastrocnemius muscle, heart, and brain were harvested. Portions of the liver and muscle were analyzed by immunoblotting for phospho-S235/236 S6, S6, phospho-S65 4E-BP1, and 4E-BP1 levels as described[18] except that tissues were disrupted with a homogenizer instead of a sonicator. Small portions of the liver, heart, and brain were fixed in formalin and processed for phospho-S235/236 S6 immunohistochemistry as described[28]. All mice were housed in a barrier facility with standard 12-h light/dark cycle. Food and water were available *ad libitum* until the 8-hour fast. Animal studies were performed under AAALAC and NIH guidelines and were approved by institutional animal care and use committee (IACUC). No randomization or blinding was performed.

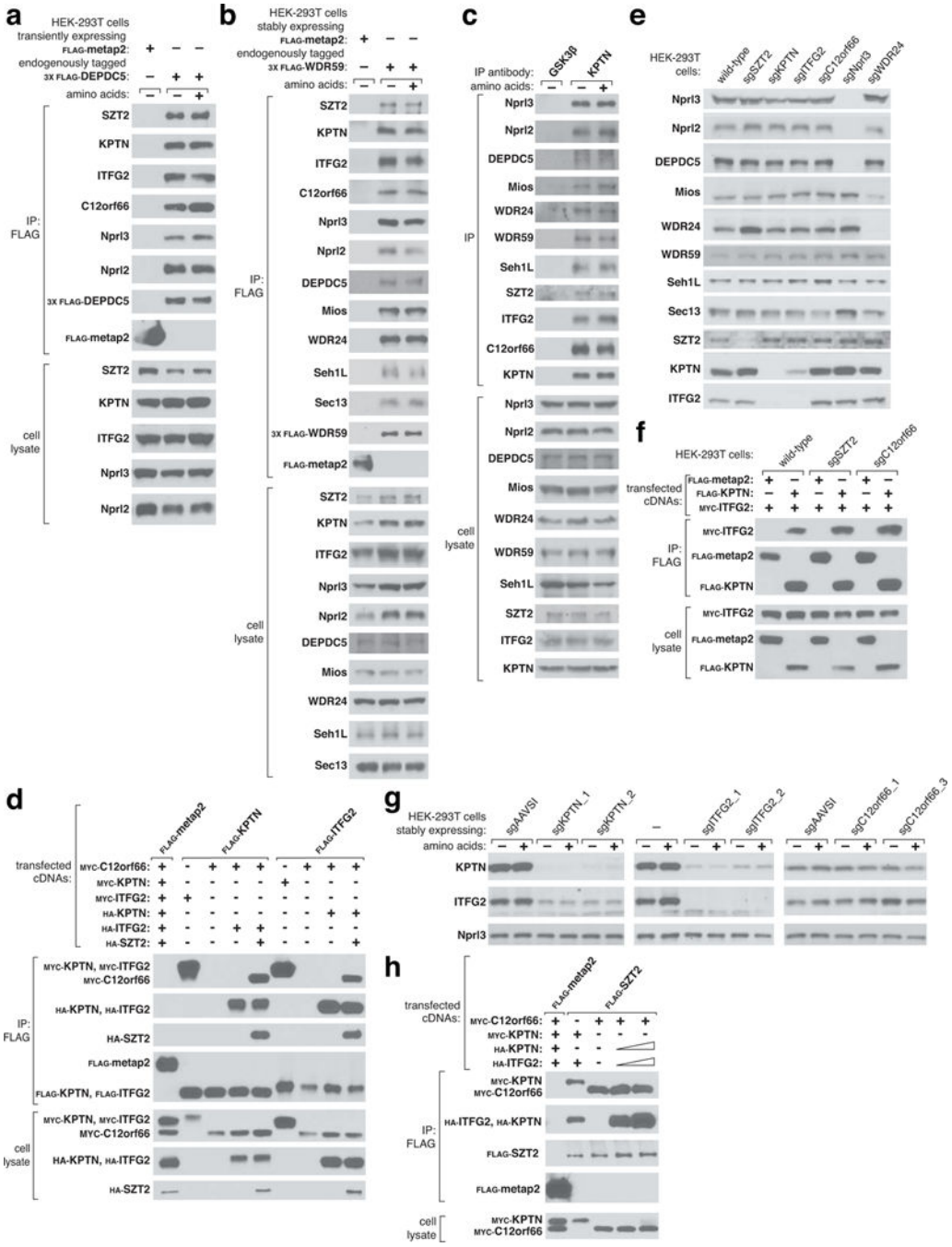
### Size exclusion chromatography

A confluent 15 cm plate of wild-type or SZT2-defiicient HEK-293T cells (~40 × $10^6$ cells) was lysed in a CHAPS-containing buffer (50 mM NaHEPES, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, and 0.3% CHAPS). The lysate was cleared by ultracentrifugation at 100,000 ×g for 30 minutes, to completely remove non-lysed cells and insoluble aggregates. The cleared lysate was normalized to a protein concentration of 3 mg/ml, and 1 ml of it was loaded onto a tandem Superose 6 gel filtration column. The elutant was fractionated and the proteins in each fraction were precipitated separately with trichlorocacetic acid (TCA). The precipitants were collected by centrifugation and washed twice with acetone to remove residual TCA. The protein pellets were re-dissolved in 2xSDS loading buffer. Equal amounts of each were separated by 8% SDS-PAGE and analyzed by immunoblotting for indicated antibodies.

### Data availability statement

No large-scale datasets were generated or analysed during the current study. Plasmids that were generated are available on Addgene.

## Extended Data




**Extended Data Figure 1. GATOR1 and GATOR2 associate with endogenous KICSTOR components in an amino acid insensitive fashion**

a) An endogenously tagged GATOR1 component co-immunoprecipitates endogenous KICSTOR. Anti-FLAG immunoprecipitates were prepared from HEK-293T cells expressing endogenously FLAG-tagged DEPDC5, a GATOR1 component, that had been starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min.

Immunoprecipitates and cell lysates were analyzed by immunoblotting for the indicated proteins.

b) An endogenously tagged GATOR2 component co-immunoprecipitates endogenous GATOR1 and KICSTOR. Anti-FLAG immunoprecipitates were prepared from HEK-293T cells expressing endogenously FLAG-tagged WDR59, a GATOR2 component, and treated as in (a). Immunoprecipitates and cell lysates were analyzed by immunoblotting for the indicated proteins.

c) An anti-KPTN antibody co-immunoprecipitates endogenous components of KICSTOR, GATOR1, and GATOR2. Anti-KPTN immunoprecipitates were prepared from wild-type HEK-293T treated as in (a) and immunoprecipitates and cell lysates analyzed by immunoblotting for the indicated proteins. Anti-GSK3β immunoprecipitates were used to monitor the non-specific binding of proteins to the beads.

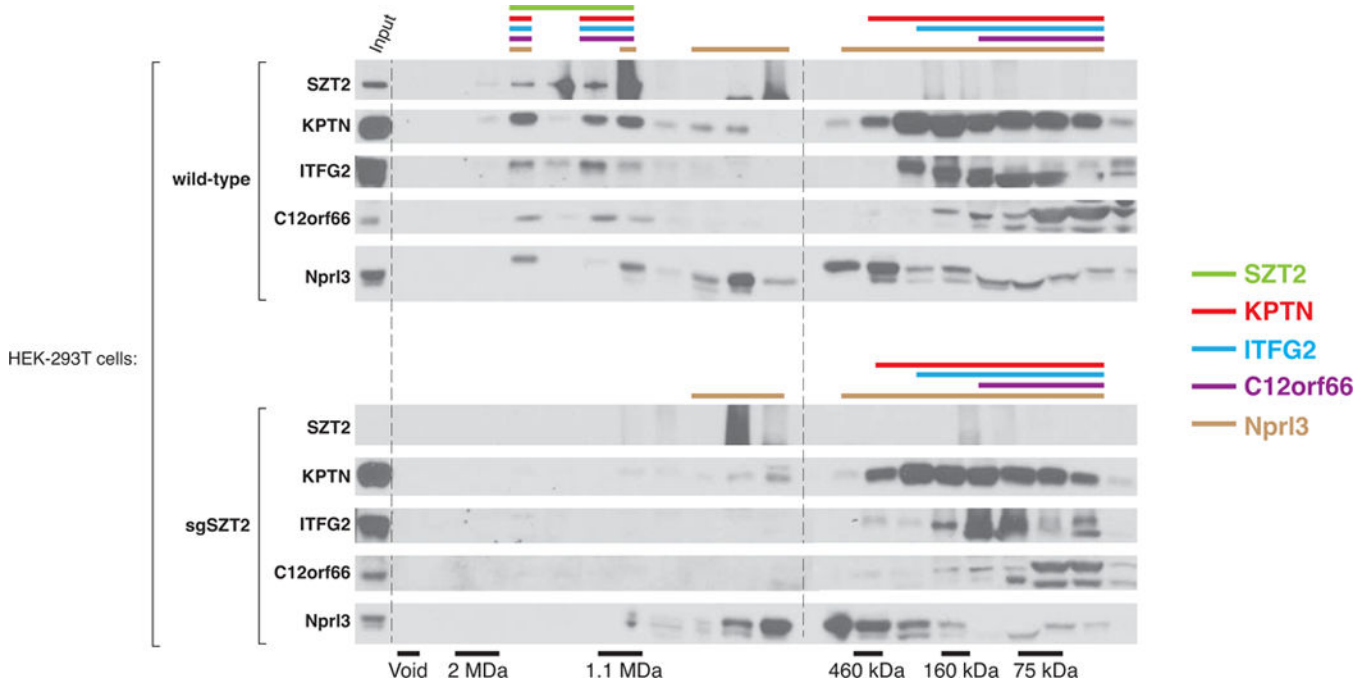
d) KPTN and ITFG2 form a heterodimer that requires SZT2 to associate with C12orf66. Anti-FLAG immunoprecipitates and lysates prepared from HEK-293T cells expressing the indicated cDNAs were analyzed by immunoblotting for the relevant epitope tags.

e) Loss of KICSTOR components did not have a significant effect on the expression levels of GATOR1 or GATOR2 components. HEK-293T cell clones deficient for indicated KICSTOR components or Nprl3 or WDR24 were generated via the CRISPR/Cas9 system and single cell cloning. Cell lysates were analyzed by immunoblotting for the indicated proteins. DNA sequencing of the *C12orf66* gene was used to verify out of frame mutations in the genomic locus of the sgC12orf66 cells because an antibody that detects the C12orf66 protein in cell lysates is not available. The HEK-293T cell clones analyzed here were used in subsequent figures, where indicated.

f) KPTN interacts with ITFG2 even in cells lacking other KICSTOR components. Immunoprecipitates and cell lysates prepared from wild-type, SZT2-deficient, or C12orf66-deficient HEK-293T cell clones expressing the indicated proteins were analyzed by immunoblotting.

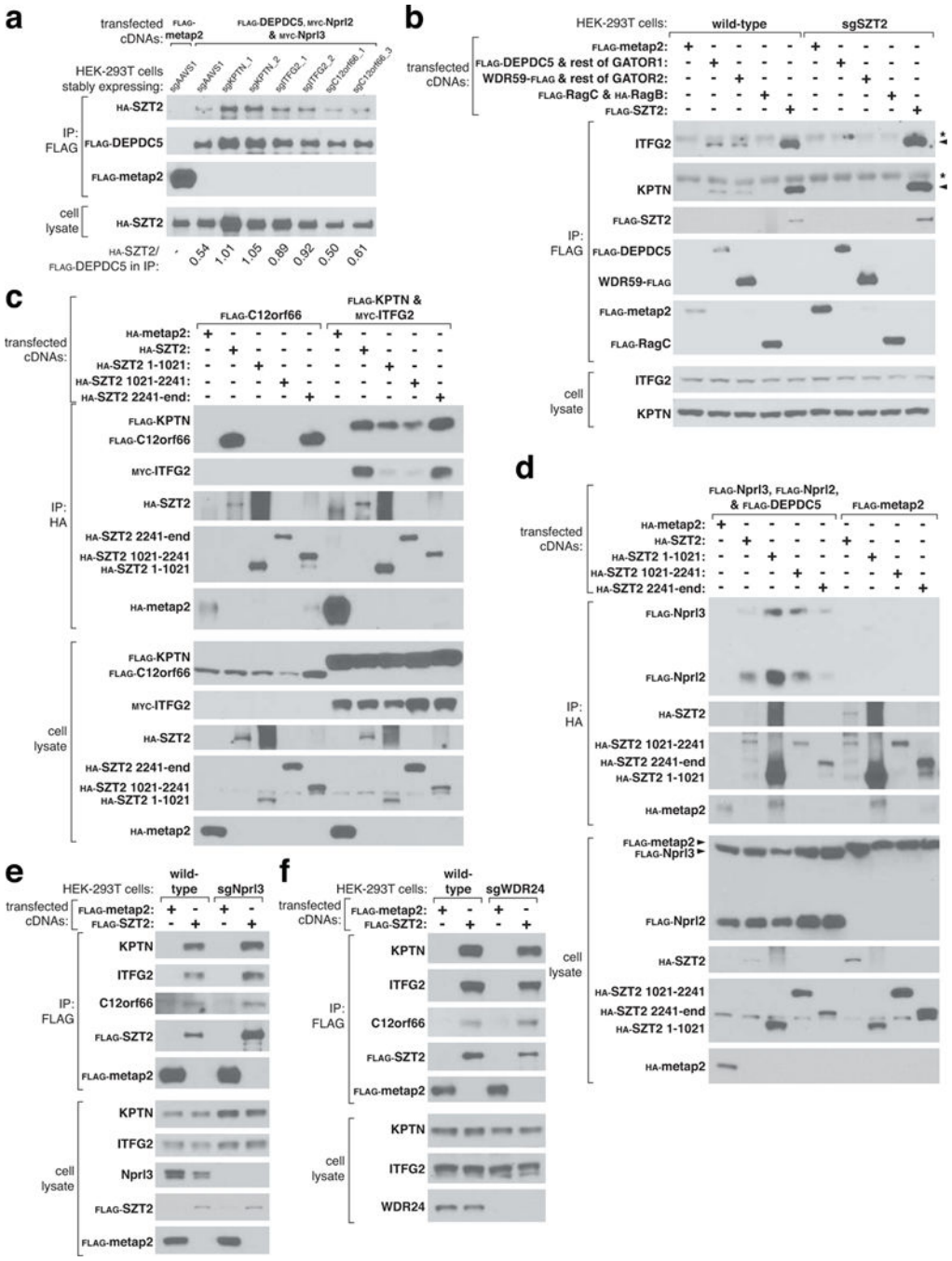
g) Expression levels of KPTN and ITFG2 in HEK-293T cells stably expressing the indicated sgRNAs under amino acid starved or replete conditions. Cell lysates were analyzed by immunoblotting for the levels of the indicated proteins. Raptor serves as a loading control. These same cell lines were also analyzed for phospho-S6K1 and S6K1 levels in Extended Data Fig. 5a–c.

h) KPTN-ITFG2 does not compete with C12orf66 for associating with SZT2. HEK-293T cells expressing the indicated cDNAs were treated and analyzed as in (d).




**Extended Data Figure 2. Size exclusion chromatography analysis of KICSTOR components**

Lysates of wild-type or SZT2-deficient (sgSZT2) HEK-293T cells were fractionated with tandem Superose 6 size exclusion chromatography columns and the collected fractions analyzed by immunoblotting for the indicated proteins. Colored bars indicate fractions that contain the protein denoted by that color in the key. Fractions containing the molecular weight standards are indicated. Note that the C12orf66 antibody exhibits significant background when used to probe total cell lysates by immunoblotting so we are only confident that the bands in the high molecular weight fractions that disappear in the SZT2-deficient cells actually represent C12orf66.




**Extended Data Figure 3. SZT2 interacts with KPTN-ITFG2, C12orf66, and GATOR1**

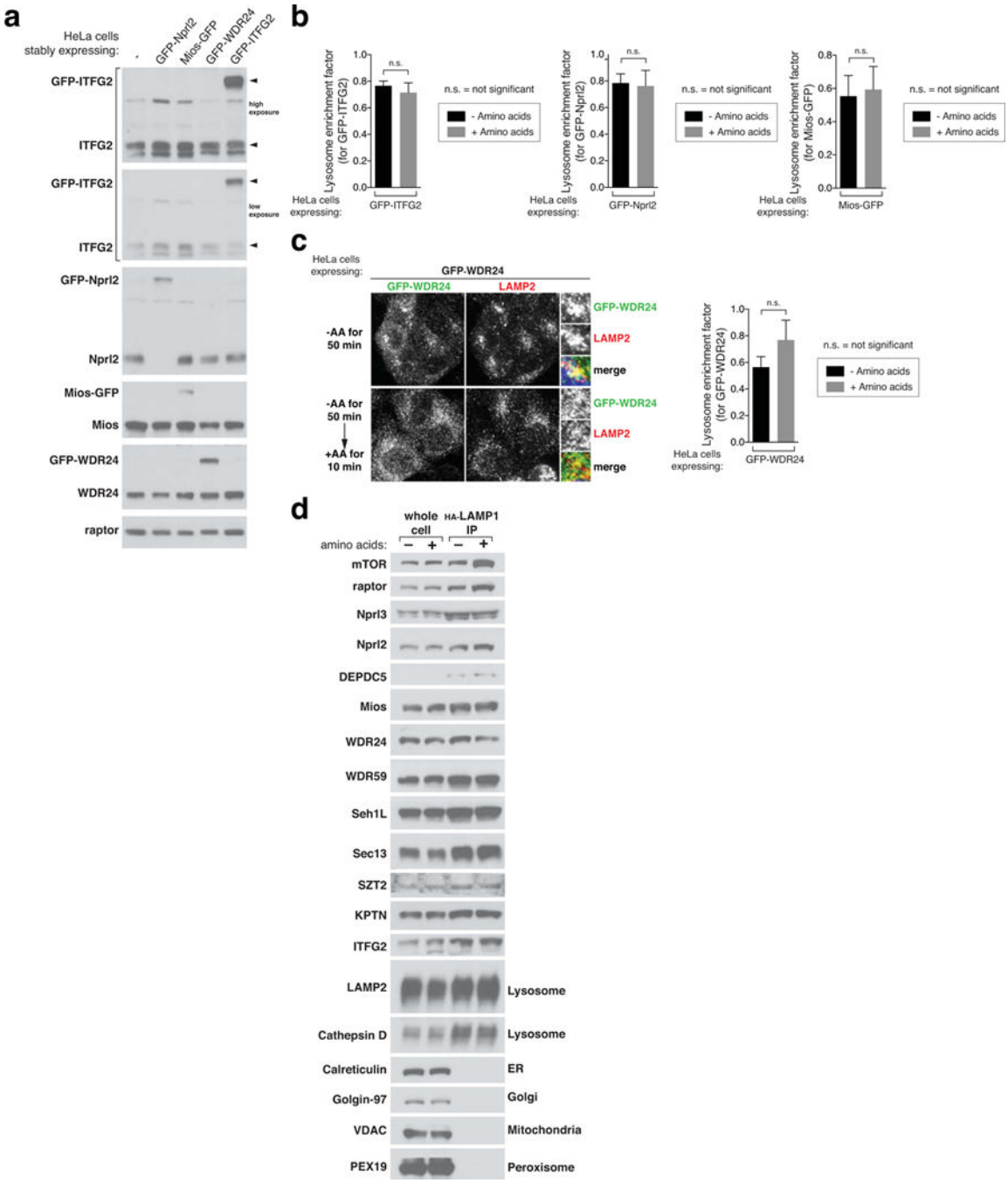
a) SZT2 interacts with GATOR1 in the absence of other KICSTOR components. HEK-293T cells stably expressing a control sgRNA (sgAAVS1) or sgRNAs targeting the indicated KICSTOR components were transfected with the indicated cDNAs. Anti-FLAG immunoprecipitates were prepared and analyzed, along with cell lysates, by immunoblotting for the relevant epitope tags. The ratios of the intensities of the HA-SZT2 to FLAG-DEPDC5 bands are indicated below the FLAG-DEPDC5 blot. See Extended Data Fig. 1g for the expression levels of the KICSTOR components in the cell lines used here.

b) SZT2 links the other KICSTOR components to the GATOR complexes. Anti-FLAG immunoprecipitates prepared from wild-type or SZT2-deficient HEK-293T cells expressing the indicated cDNAs were analyzed by immunoblotting for the indicated proteins. * marks non-specific bands. See Extended Data Fig. 1e for the expression level of SZT2 in the SZT2-deficient HEK-293T cells.

c) C12orf66 interacts with SZT2 at a distinct site than KPTN-ITFG2. HEK-293T cells expressing the indicated cDNAs were analyzed as in (b).

d) GATOR1 interacts with the first and second regions of SZT2. HEK-293T cells expressing the indicated cDNAs were analyzed as in (b).

e) The association of SZT2 with KPTN-ITFG2 persists in the absence of GATOR1 or (f) GATOR2. Anti-FLAG immunoprecipitates were prepared from wild-type, Nprl3-deficient, or WDR24-deficient HEK-293T cells expressing the indicated cDNAs and analyzed as in (b).




**Extended Data Figure 4. Subcellular localization of the GATOR and KICSTOR complexes**

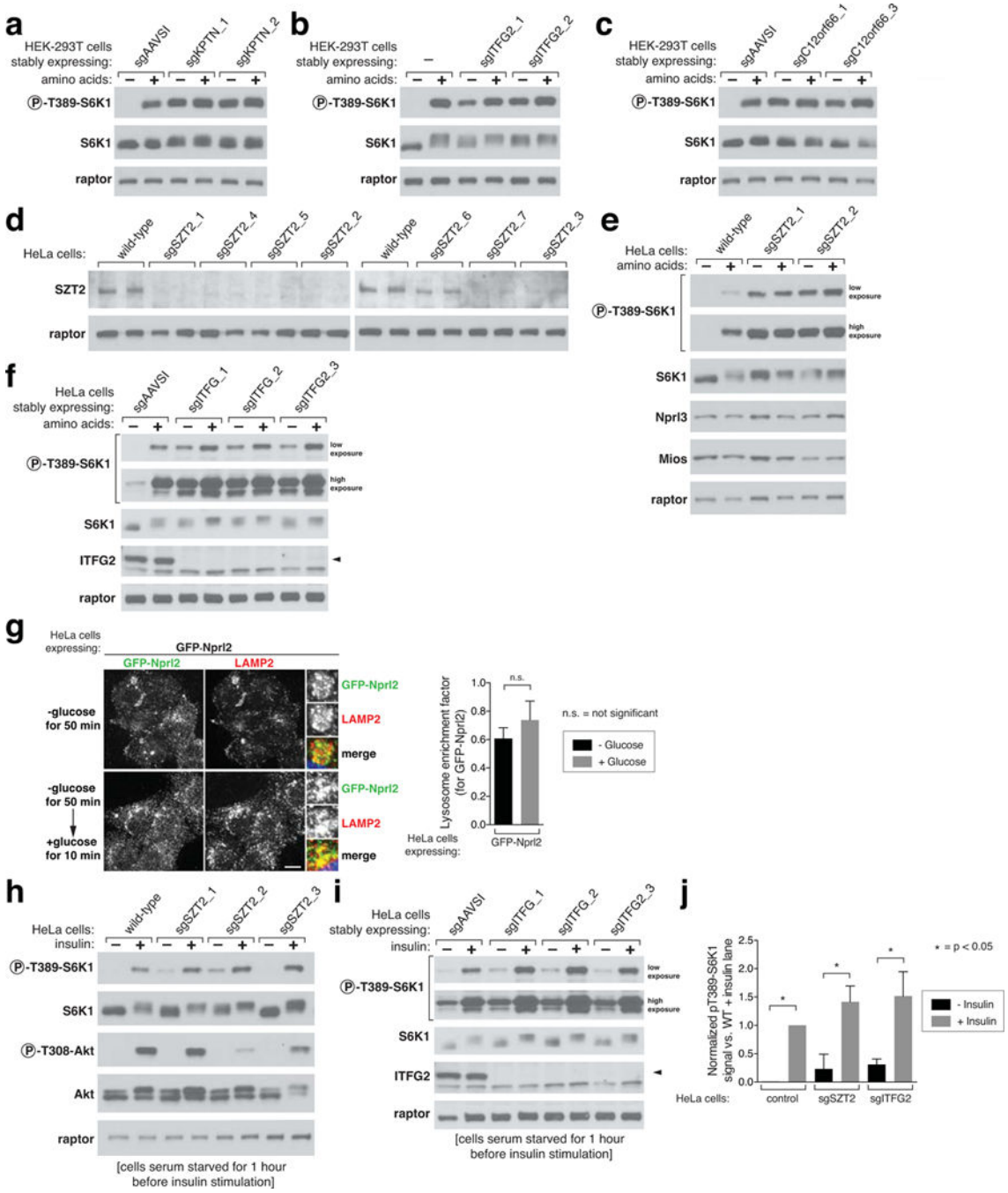
a) Expression levels of GFP-tagged GATOR1, GATOR2, or KICSTOR components used in the localization experiments. Nprl2-deficient or wild-type HeLa cells stably expressing the indicating GFP-tagged proteins were single cell sorted for the low GFP population and single cell clones were analyzed by immunoblotting for levels of the indicated proteins.

b) Quantitation of the imaging data in Figures 1g, h, i. Values are mean ± standard error.

c) Amino acids do not control the localization of GATOR2 to the lysosomal surface. Wild-type HeLa cells stably expressing GFP-tagged WDR24, a component of GATOR2, were

starved or starved and restimulated with amino acids for the indicated times prior to processing for immunofluorescence detection of GFP and LAMP2. Scale bars represent 10 μm. Quantitation of the imaging data is shown in the bar graph on the right.

d) Amino acids do not regulate the amounts of GATOR1, GATOR2, or KICSTOR components on purified lysosomes. Lysosomes immunopurified with anti-HA beads from wild-type HEK-293T cells expressing HA-tagged LAMP1 and treated as in (c) were analyzed by immunoblotting for the levels of the indicated proteins.



**Extended Data Figure 5. KICSTOR loss affects the sensitivity of the mTORC1 pathway to nutrients but not growth factors**

a) CRISPR/Cas9-mediated depletion of KPTN, (b) ITFG2, or (c) C12orf66 renders mTORC1 signaling insensitive to amino acid deprivation. HEK-293T cells stably expressing the indicated sgRNAs were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the indicated proteins. See Extended Data Fig. 1g for the expression levels of the KICSTOR components in the cell lines used here.

d) Testing of indicated SZT2 sgRNA-treated HeLa cell clones for levels of SZT2 and raptor by immunoblotting. Note that not all clones have complete loss of the SZT2 protein.

e) mTORC1 signaling in SZT2-deficient cells is insensitive to amino acid deprivation. Indicated HeLa cell SZT2-deficient clones from (d) were treated and analyzed as in (a).

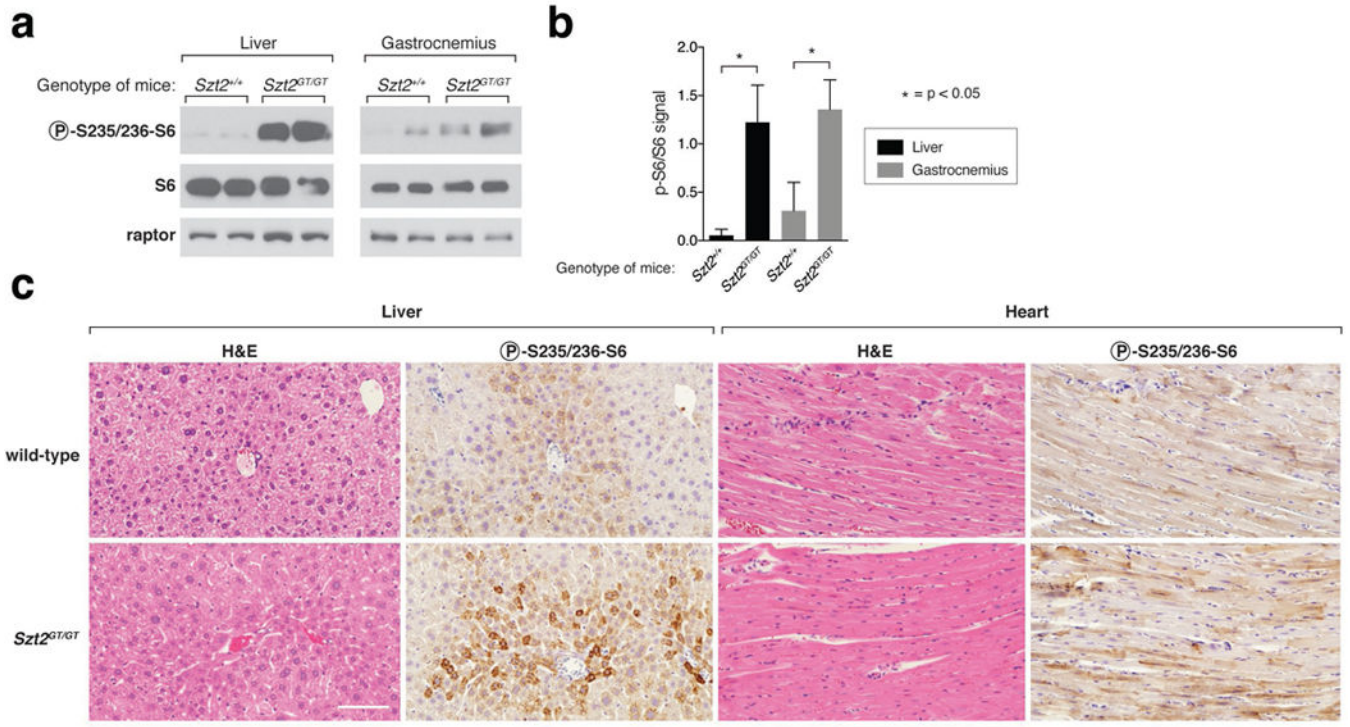
f) CRISPR/Cas9-mediated depletion of ITFG2 renders mTORC1 signaling partially insensitive to amino acid deprivation. HeLa cells stably expressing the indicated ITFG2 sgRNAs were treated and analyzed as in (a).

g) Glucose levels do not affect GATOR1 localization, as monitored by GFP-Nprl2. HeLa cells expressing GFP-Nprl2 from Figure 1h were starved of glucose for 50 min or starved and restimulated with glucose for 10 min prior to processing for immunofluorescence for GFP and LAMP2 (left). Scale bars represent 10 μm. Quantitation of the imaging data is shown in bar graph on the right.

h) In SZT2-deficient cells mTORC1 signaling is still sensitive to serum starvation and insulin stimulation. Indicated HeLa cell SZT2-deficient clones from (d) were starved of serum for 50 min or starved of serum and restimulated with insulin for 10 min. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the indicated proteins.

i) In cells with CRISPR/Cas9-mediated depletion of ITFG2, mTORC1 signaling is still sensitive to serum starvation and insulin stimulation. HeLa cells stably expressing the indicated ITFG2 sgRNAs were treated and analyzed as in (h).

j) Quantitation of phospho-S6K1 blots in (h) and (i). Values are mean ± standard deviation.

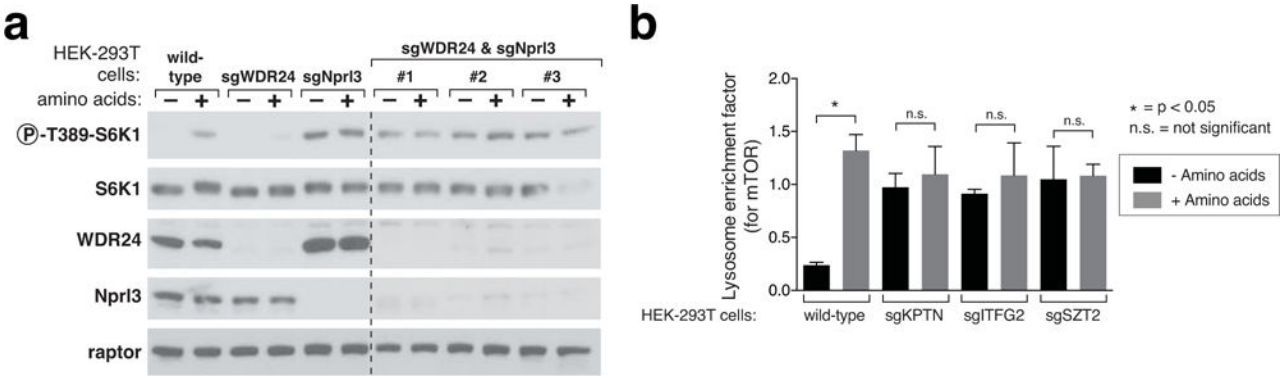



**Extended Data Figure 6. Activation of mTORC1 signaling in tissues from *Szt2*$^{GT/GT}$ mice**

a) SZT2 inhibits mTORC1 signaling in mouse liver and muscle. Mice with the indicated genotypes were treated and analyzed by immunoblotting for the levels and phosphorylation state of S6 as in Figure 2d. The animals examined here represent an additional two animals of each genotype beyond those analyzed in Figures 2d, e.

b) Quantitation of the ratio of phospho-S6 to S6 bands in (a) and in Figures 2d, e. Values are mean ± standard deviation for n=4.

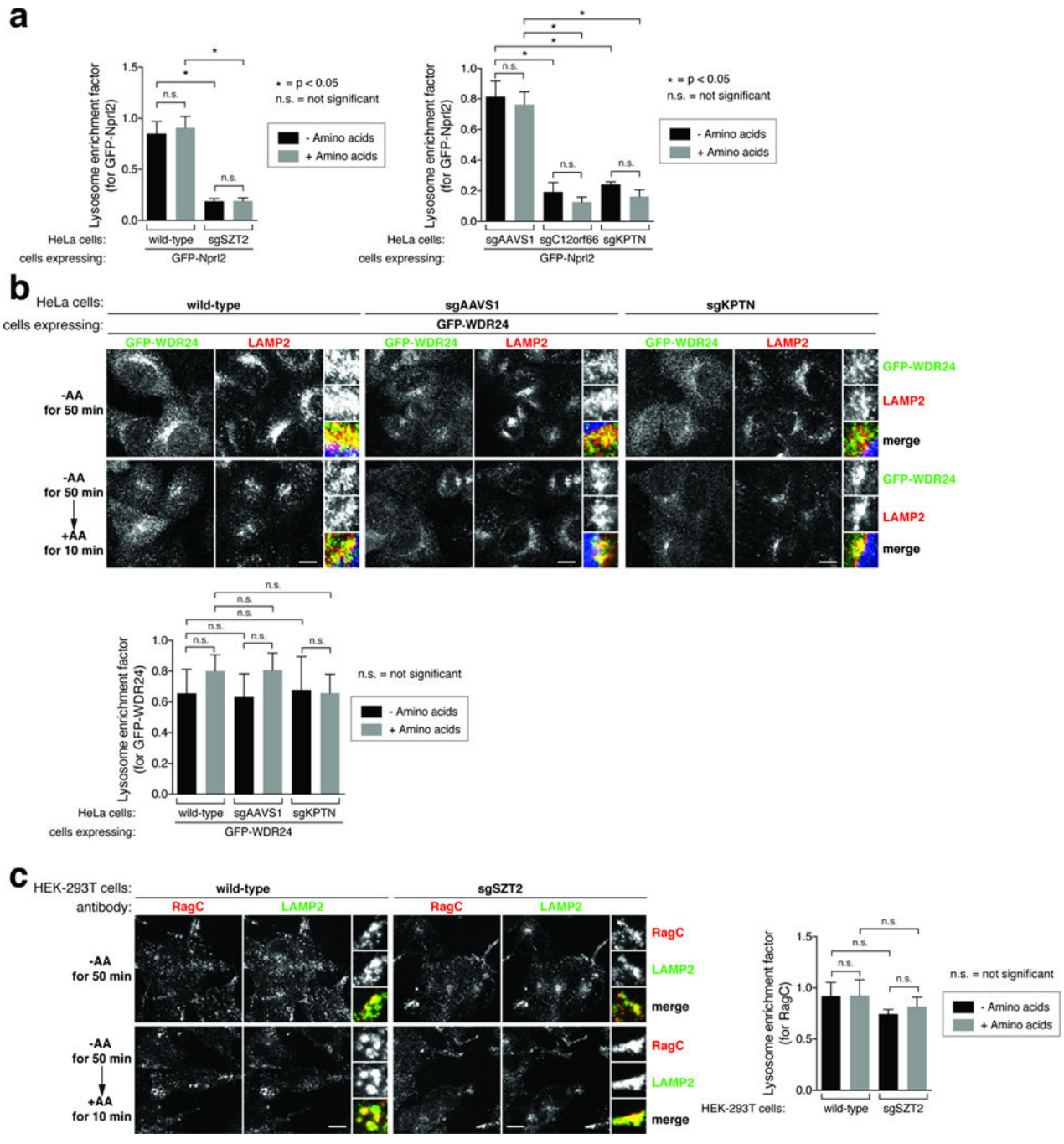
c) SZT2 inhibits mTORC1 signaling in hepatocytes and cardiomyocytes in *vivo*. Liver and heart sections prepared from mice treated as in Figure 2d were analyzed by immunohistochemistry for phospho-S235/236 S6 levels and serial sections were stained with hematoxylin and eosin (H&E). Liver image is centered over a central vein. Scale bar represents 40 μm.



**Extended Data Figure 7. GATOR1, like KICSTOR, functions downstream of or parallel to GATOR2 in the mTORC1 pathway**

a) GATOR1, like KICSTOR, is epistatic to GATOR2. Wild-type, WDR24-deficient, or Nprl3- and WDR24-deficient HEK-293Ts were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Cell lysates were analyzed by immunoblotting for the indicated proteins and phosphorylation states.

b) Quantitation of the imaging data in Figure 3d. Values are mean ± standard error.

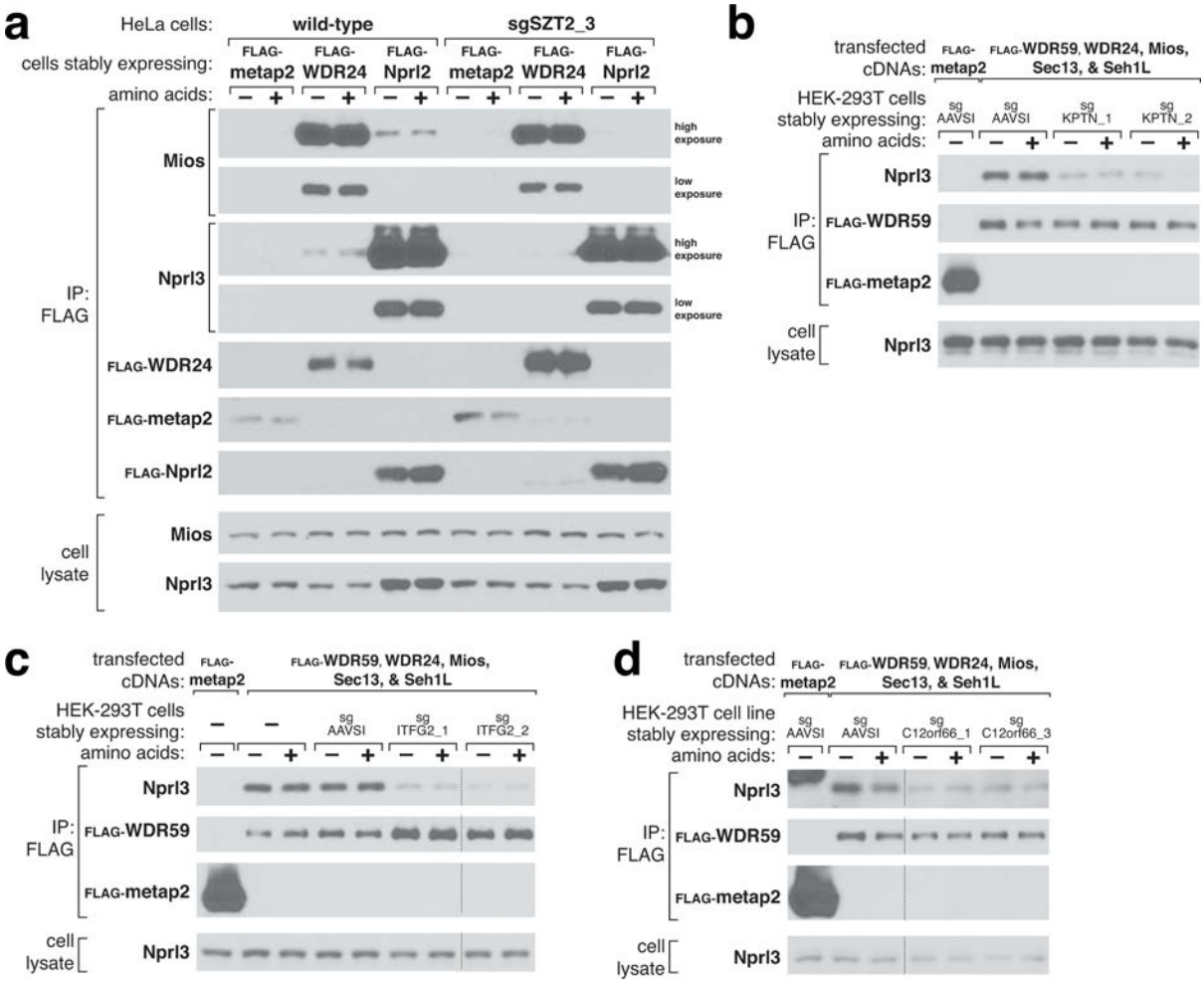


**Extended Data Figure 8. KICSTOR regulates the lysosomal localization of GATOR1 but not of GATOR2 or the Rag GTPases**

a) Quantitation of the imaging data in Figures 4a, b. Values are mean ± standard error.

b) Loss of KICSTOR components does not affect the lysosomal localization of GATOR2. GFP-WDR24 expressing HeLa cells prepared as in Extended Data Fig. 4c were subsequently modified with the CRISPR/Cas9 system to create KPTN-deficient cells. These cells as well as wild-type and sgAAVS1-treated control cells were starved or starved and restimulated with amino acids for the noted times prior to processing for the detection of GFP and LAMP2 by immunofluorescence. Insets depict selected fields magnified 3.24× and their overlays. Scale bars represent 10 μm. Quantitation of the imaging data is shown in the bar graph below images (mean ± standard error).

c) The Rag GTPases localize to lysosomes regardless of SZT2 expression. Wild-type and SZT2-deficient HEK-293T cells were treated as in (b) prior to processing for the detection of RagC and LAMP2 by immunofluorescence. Insets depict selected fields magnified 3.24× and their overlays. Scale bars represent 10 μm. Quantitation of the imaging data is shown in the bar graph on the right (mean ± standard error).




**Extended Data Figure 9. Loss of KICSTOR disrupts the GATOR1-GATOR2 interaction**

a) Loss of SZT2 disrupts the GATOR1-GATOR2 interaction. Anti-FLAG immunoprecipitates were prepared from wild-type or SZT2-deficient HeLa cells stably expressing the indicated cDNAs and starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Immunoprecipitates and cell lysates were analyzed by immunoblotting for the indicated proteins.

b) KPTN is necessary for the interaction of GATOR2 with GATOR1. HEK-293T cells stably expressing the indicated sgRNAs were transfected with the indicated cDNAs and subsequently treated and analyzed as in (a).

c) ITFG2 is also necessary for the GATOR1-GATOR2 interaction. Cells were prepared, treated, and analyzed as in (b).

d) C12orf66 is also necessary for the GATOR1-GATOR2 interaction. Cells were prepared, treated, and analyzed as in (b).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank all members of the Sabatini Lab for helpful insights. We thank Kara McKinley and Iain Cheeseman for generously providing the retroviral GFP constructs used in this work, Connie Mahaffey for technical support with the mouse experiments, and Jianxin Xie from Cell Signaling Technology, Inc. for generously providing the DEPDC5, Mios, Nprl2, WDR24, WDR59, C12orf66, Seh1L, and SZT2 antibodies. This work was supported by grants from the NIH to D.M.S (R01 CA103866 and R37 AI47389) and W.N.F. (R37 NS031348), Department of Defense (W81XWH-07-0448) to D.M.S., and fellowship support from the NIH to R.L.W. (T32 GM007753 and F30 CA189333), L.C. (F31 CA180271), and J.M.O (T32 GM007753 and F30 CA210373), from NSF to K.J.C. (2016197106), from the National Defense Science & Engineering Graduate Fellowship (NDSEG) Program to G.A.W., from the Life Sciences Research Foundation to K.S. (Pfizer fellow), from the EMBO Long-Term Fellowship to M.A.R, and from the Paul Gray UROP Fund to S.M.S. (3143900). D.M.S. is an investigator of the Howard Hughes Medical Institute.

## References

1. Laplante M, Sabatini DM. mTOR Signaling in Growth Control and Disease. Cell. 2012; 149:274–293. DOI: 10.1016/j.cell.2012.03.017 [PubMed: 22500797]
2. Dibble CC, Manning BD. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. Nature Cell Biology. 2013; 15:555–564. DOI: 10.1038/ncb2763 [PubMed: 23728461]
3. Jewell JL, Russell RC, Guan KL. Amino acid signalling upstream of mTOR. Nat Rev Mol Cell Biol. 2013; 14:133–139. [PubMed: 23361334]
4. Sancak Y, et al. The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to mTORC1. Science. 2008; 320:1496–1501. DOI: 10.1126/science.1157535 [PubMed: 18497260]
5. Basel-Vanagaite L, et al. Biallelic SZT2 mutations cause infantile encephalopathy with epilepsy and dysmorphic corpus callosum. Am J Hum Genet. 2013; 93:524–529. [PubMed: 23932106]
6. Venkatesan C, Angle B, Millichap JJ. Early-life epileptic encephalopathy secondary to SZT2 pathogenic recessive variants. Epileptic Disord. 2016; 18:195–200. [PubMed: 27248490]
7. Baple EL, et al. Mutations in KPTN cause macrocephaly, neurodevelopmental delay, and seizures. Am J Hum Genet. 2014; 94:87–94. [PubMed: 24239382]
8. Pajusalu S, Reimand T, Õunap K. Novel homozygous mutation in KPTN gene causing a familial intellectual disability-macrocephaly syndrome. Am J Med Genet A. 2015; 167A:1913–1915. [PubMed: 25847626]
9. Mc Cormack A, et al. 12q14 Microdeletions: Additional Case Series with Confirmation of a Macrocephaly Region. Case Rep Genet. 2015; 2015:192071. [PubMed: 26266063]

10. Crino PB. mTOR: A pathogenic signaling pathway in developmental brain malformations. Trends Mol Med. 2011; 17:734–742. [PubMed: 21890410]
11. D'Gama AM, et al. Mammalian target of rapamycin pathway mutations cause hemimegalencephaly and focal cortical dysplasia. Ann Neurol. 2015; 77:720–725. [PubMed: 25599672]
12. Baldassari S, Licchetta L, Tinuper P, Bisulli F, Pippucci T. GATOR1 complex: the common genetic actor in focal epilepsies. J Med Genet. 2016; 53:503–510. [PubMed: 27208208]
13. Sancak Y, et al. Ragulator-Rag Complex Targets mTORC1 to the Lysosomal Surface and Is Necessary for Its Activation by Amino Acids. Cell. 2010; 141:290–303. DOI: 10.1016/j.cell.2010.02.024 [PubMed: 20381137]
14. Wei Y, Lilly MA. The TORC1 inhibitors Nprl2 and Nprl3 mediate an adaptive response to amino-acid starvation in Drosophila. Cell Death Differ. 2014; 21:1460–1468. [PubMed: 24786828]
15. Wei Y, et al. TORC1 regulators Iml1/GATOR1 and GATOR2 control meiotic entry and oocyte development in Drosophila. Proceedings of the National Academy of Sciences. 2014; 111:E5670–5677.
16. Cai W, Wei Y, Jarnik M, Reich J, Lilly MA. The GATOR2 Component Wdr24 Regulates TORC1 Activity and Lysosome Function. PLoS Genet. 2016; 12:e1006036. [PubMed: 27166823]
17. Bar-Peled L, et al. A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. Science. 2013; 340:1100–1106. [PubMed: 23723238]
18. Efeyan A, et al. Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. Nature. 2013; 493:679–683. [PubMed: 23263183]
19. Kalender A, et al. Metformin, Independent of AMPK, Inhibits mTORC1 in a Rag GTPase-Dependent Manner. Cell Metabolism. 2010; 11:390–401. DOI: 10.1016/j.cmet.2010.03.014 [PubMed: 20444419]
20. Frankel WN, Yang Y, Mahaffey CL, Beyer BJ, O'Brien TP. Szt2, a novel gene for seizure threshold in mice. Genes Brain Behav. 2009; 8:568–576. [PubMed: 19624305]
21. Baulac S. mTOR signaling pathway genes in focal epilepsies. Prog Brain Res. 2016; 226:61–79. [PubMed: 27323939]
22. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell. 2002
23. Boussif O, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proceedings of the National Academy of Sciences of the United States of America. 1995; 92:7297–7301. [PubMed: 7638184]
24. Tsun ZY, et al. The Folliculin Tumor Suppressor Is a GAP for the RagC/D GTPases That Signal Amino Acid Levels to mTORC1. Molecular cell. 2013; 52:495–505. DOI: 10.1016/j.molcel.2013.09.016 [PubMed: 24095279]
25. Zoncu R, et al. mTORC1 Senses Lysosomal Amino Acids Through an Inside-Out Mechanism That Requires the Vacuolar H+-ATPase. Science. 2011; 334:678–683. DOI: 10.1126/science.1207056 [PubMed: 22053050]
26. Petit CS, Roczniak-Ferguson A, Ferguson SM. Recruitment of folliculin to lysosomes supports the amino acid-dependent activation of Rag GTPases. The Journal of Cell Biology. 2013; 202:1107–1122. [PubMed: 24081491]
27. Schindelin J, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012; 9:676–682. [PubMed: 22743772]
28. Yilmaz ÖH, et al. mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. Nature. 2012

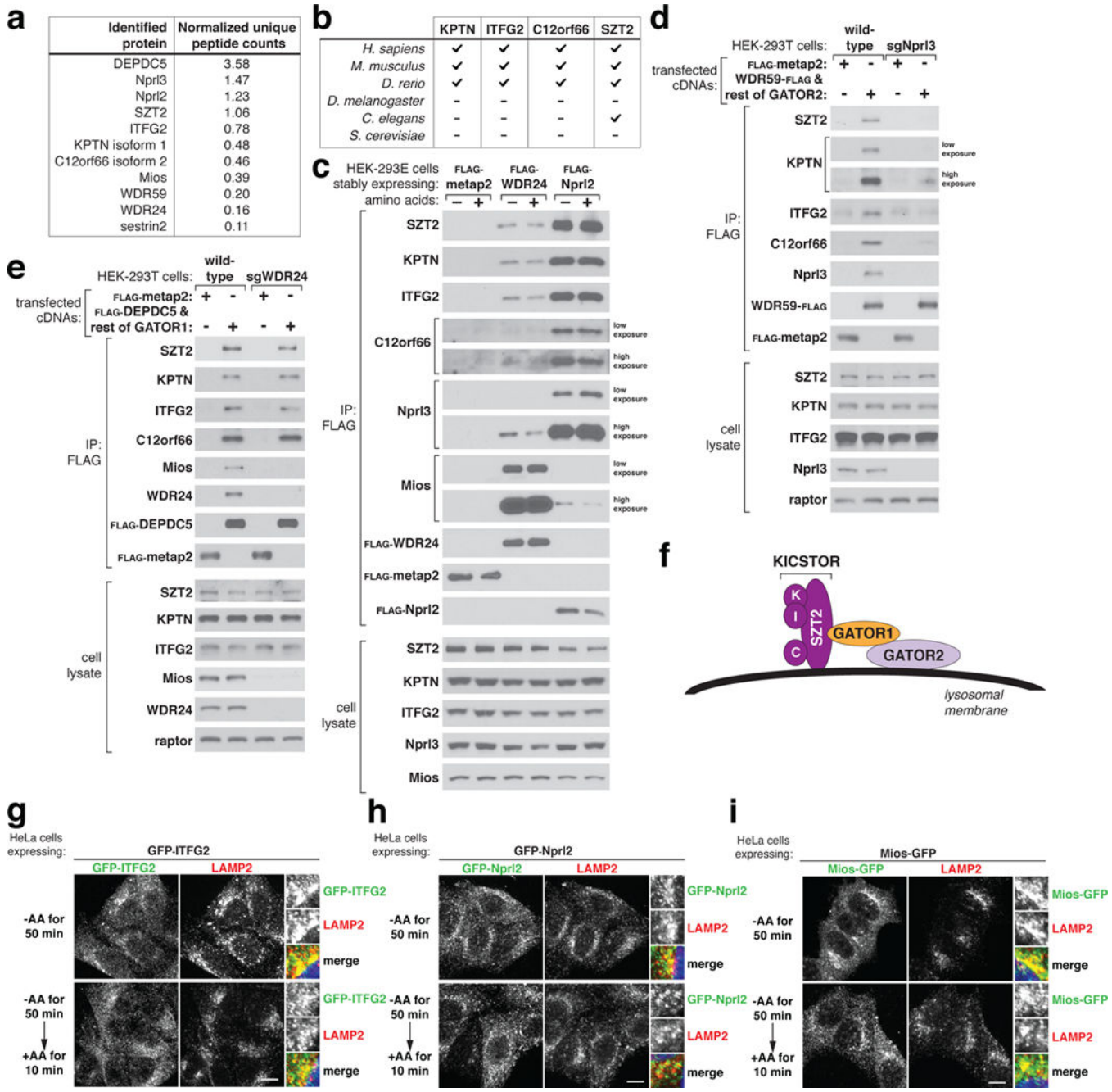



**Figure 1. The KICSTOR complex interacts with GATOR1 and localizes to lysosomes**

a) Mass spectrometric analyses identified KICSTOR-derived peptides in immunoprecipitates prepared from HEK-293T cells expressing endogenously FLAG-tagged DEPDC5.

b) Presence or absence of KICSTOR component gene orthologs in model organisms.

c) GATOR1 co-immunoprecipitates more KICSTOR than does GATOR2. Immunoprecipitates were prepared from HEK-293E cells stably expressing the indicated FLAG-tagged proteins and analyzed by immunoblotting for the indicated proteins.

d) GATOR2 requires GATOR1 to associate with KICSTOR. Anti-FLAG immunoprecipitates were prepared from wild-type and Nprl3-deficient HEK-293T cells

transiently expressing the indicated cDNAs. Anti-FLAG immunoprecipitates and lysates were analyzed as in (c).

e) GATOR1 does not require intact GATOR2 to interact with KICSTOR. Wild-type and WDR24-deficient HEK-293T cells transiently expressing the indicated cDNAs were treated and analyzed as in (c).

f) Model proposing that the KICSTOR complex interacts with GATOR1, which in turn interacts with GATOR2. KPTN, ITFG2, and C12orf66 are indicated by the first letter of each.

g) KICSTOR localizes to lysosomes in an amino acid independent fashion. Wild-type HeLa cells stably expressing GFP-ITFG2 were starved or starved and restimulated with amino acids for the indicated times prior to processing for immunofluorescence detection of GFP and LAMP2, a lysosomal marker. In all images, insets depict selected fields magnified 3.24× and their overlays. Scale bar represents 10 μm.

h) GATOR1 localizes to lysosomes regardless of amino acid levels. Nprl2-deficient HeLa cells were reconstituted with GFP-Nprl2 and treated and analyzed as in (g).

i) GATOR2 localizes to lysosomes regardless of amino acid levels. Wild-type HeLa cells stably expressing Mios-GFP were treated and analyzed as in (g).

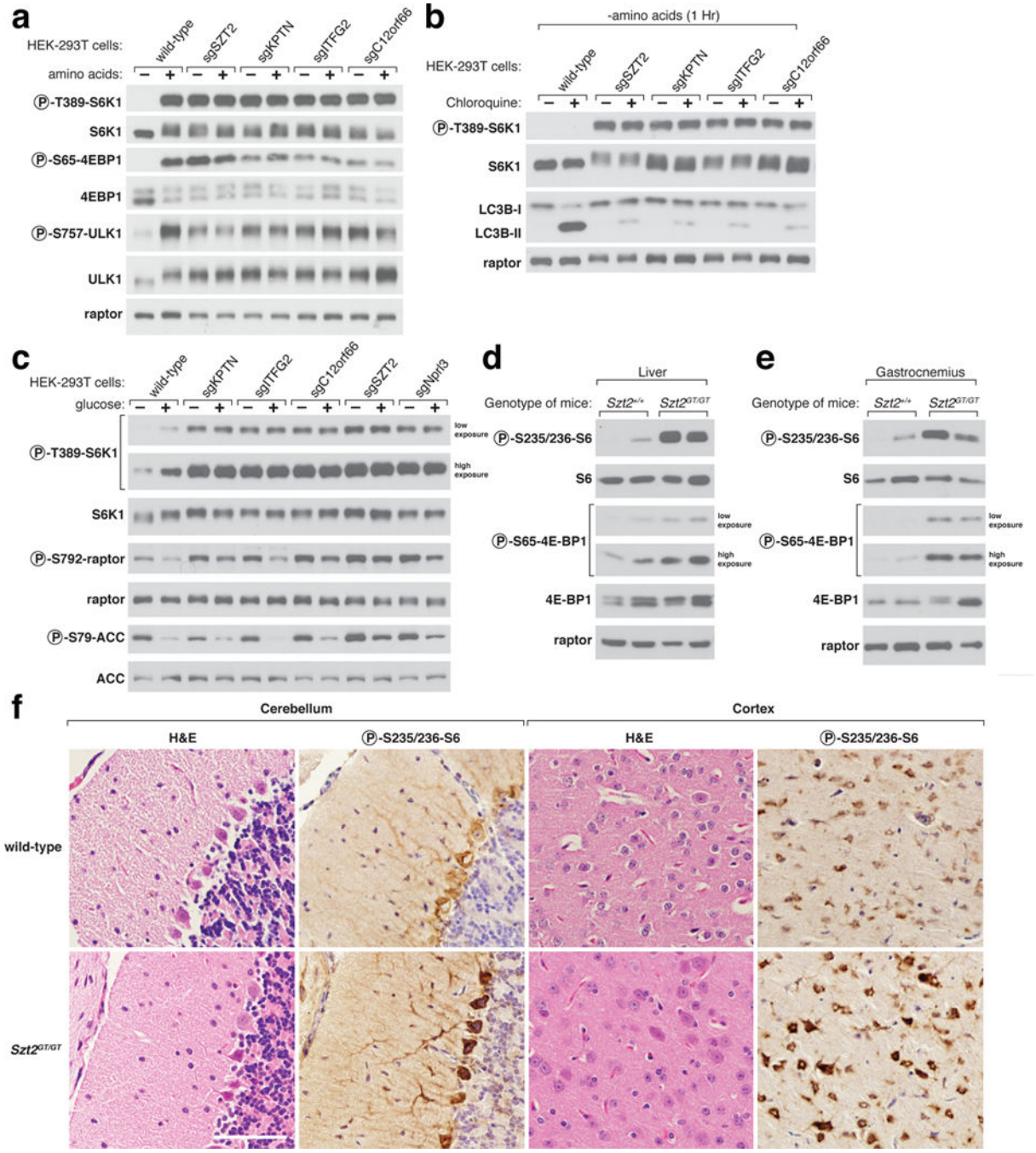



**Figure 2. Regulation of mTORC1 signaling by nutrients requires KICSTOR**

a) mTORC1 signaling is insensitive to amino acid starvation in cells lacking any KICSTOR component. HEK-293T cell clones deficient for each KICSTOR component were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the indicated proteins. See Extended Data Fig. 1e for validation of the KICSTOR deficient cells.

b) Activation of autophagy induced by amino acid starvation requires KICSTOR. The HEK-293T cell clones as in (a) were monitored for LC3B processing and accumulation after one hour of amino acid starvation in the absence or presence of chloroquine.
c) mTORC1 signaling is insensitive to glucose starvation in cells lacking KICSTOR. Experiment was performed as in (a) except that cells were starved of and stimulated with glucose.
d) SZT2 inhibits mTORC1 signaling in mouse liver. Mice with the indicated genotypes were fasted for 8 hours and liver lysates analyzed by immunoblotting for the levels and phosphorylation states of the indicated proteins. Two wild-type and two $Szt2^{GT/GT}$ mice were analyzed in this experiment and in (e).
e) SZT2 inhibits mTORC1 signaling in the mouse gastrocnemius muscle. Mice were treated and muscle lysates analyzed as in (d).
f) SZT2 inhibits mTORC1 signaling in mouse neurons *in vivo*. Brain sections prepared from mice treated as in (d) were analyzed by immunohistochemistry for phospho-S235/236 S6 levels and stained with hematoxylin and eosin (H&E). Equivalent regions of the cortex and cerebellum are shown for the two genotypes. Scale bar represents 40 μm.

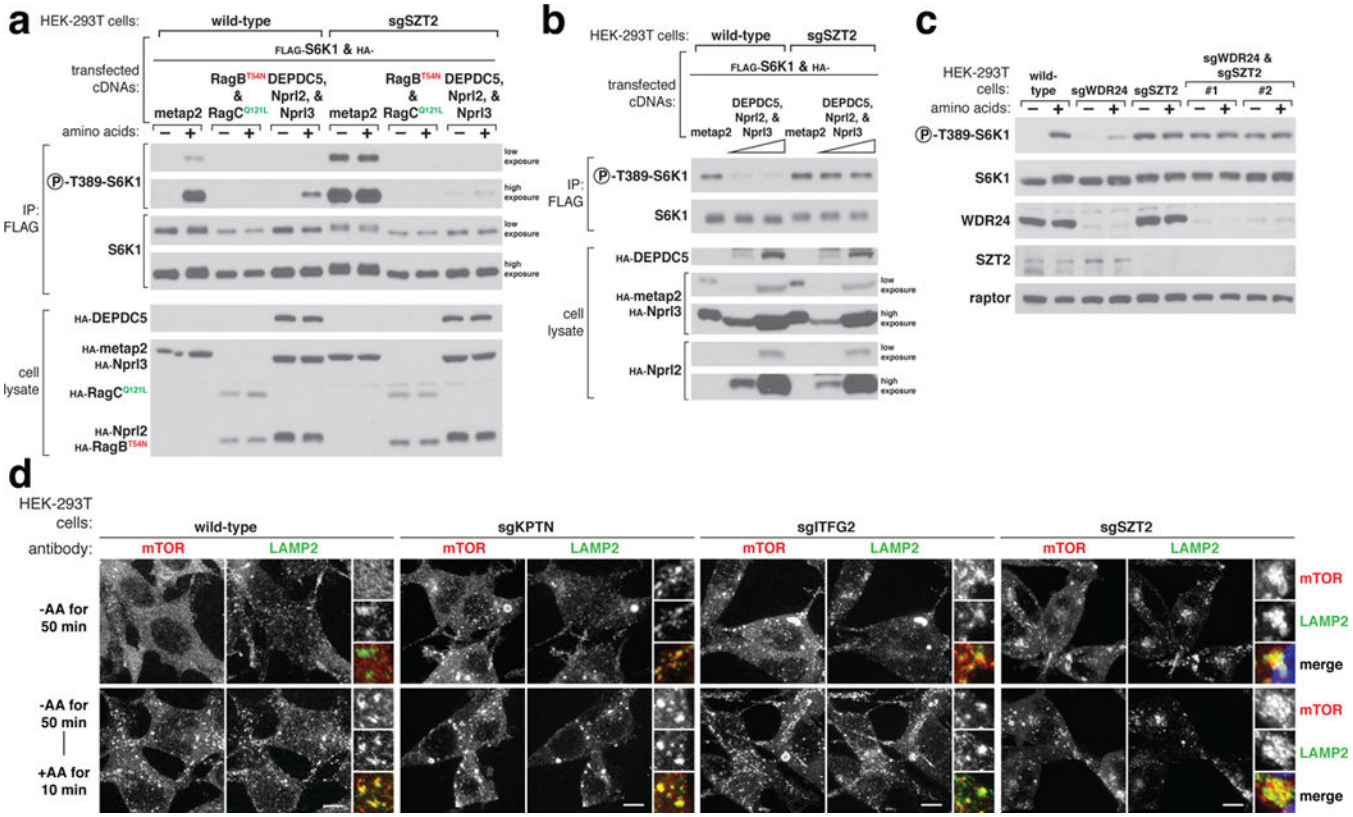



**Figure 3. KICSTOR acts upstream of the Rag GTPases**

a) KICSTOR functions upstream of the Rag GTPases. Wild-type or SZT2-deficient HEK-293Ts expressing the indicated cDNAs were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. FLAG immunoprecipitates and cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of the indicated proteins.

b) Modest GATOR1 overexpression inhibits mTORC1 signaling to a lesser extent in SZT2-deficient than in wild-type cells. FLAG immunoprecipitates and cell lysates prepared from cells expressing the indicated cDNAs and in amino acid replete conditions were analyzed as in (a). In this experiment 0.5 and 2.0 ng of the cDNA for each GATOR1 component was transfected while 100 ng of each was used in (a).

c) KICSTOR functions downstream of or in parallel to GATOR2. Wild-type, SZT2-deficient, or double SZT2- and WDR24-deficient HEK-293Ts were treated and analyzed as in (a).

d) Amino acid insensitive localization of mTOR to lysosomes in cells lacking KICSTOR components. Wild-type and KPTN-, ITFG2-, or SZT2-deficient HEK-293T cells were starved or starved and restimulated with amino acids for the indicated times prior to processing for immunofluorescence detection of mTOR and LAMP2. In all images, insets depict selected fields magnified 3.24× and their overlays. Scale bars represent 10 μm.

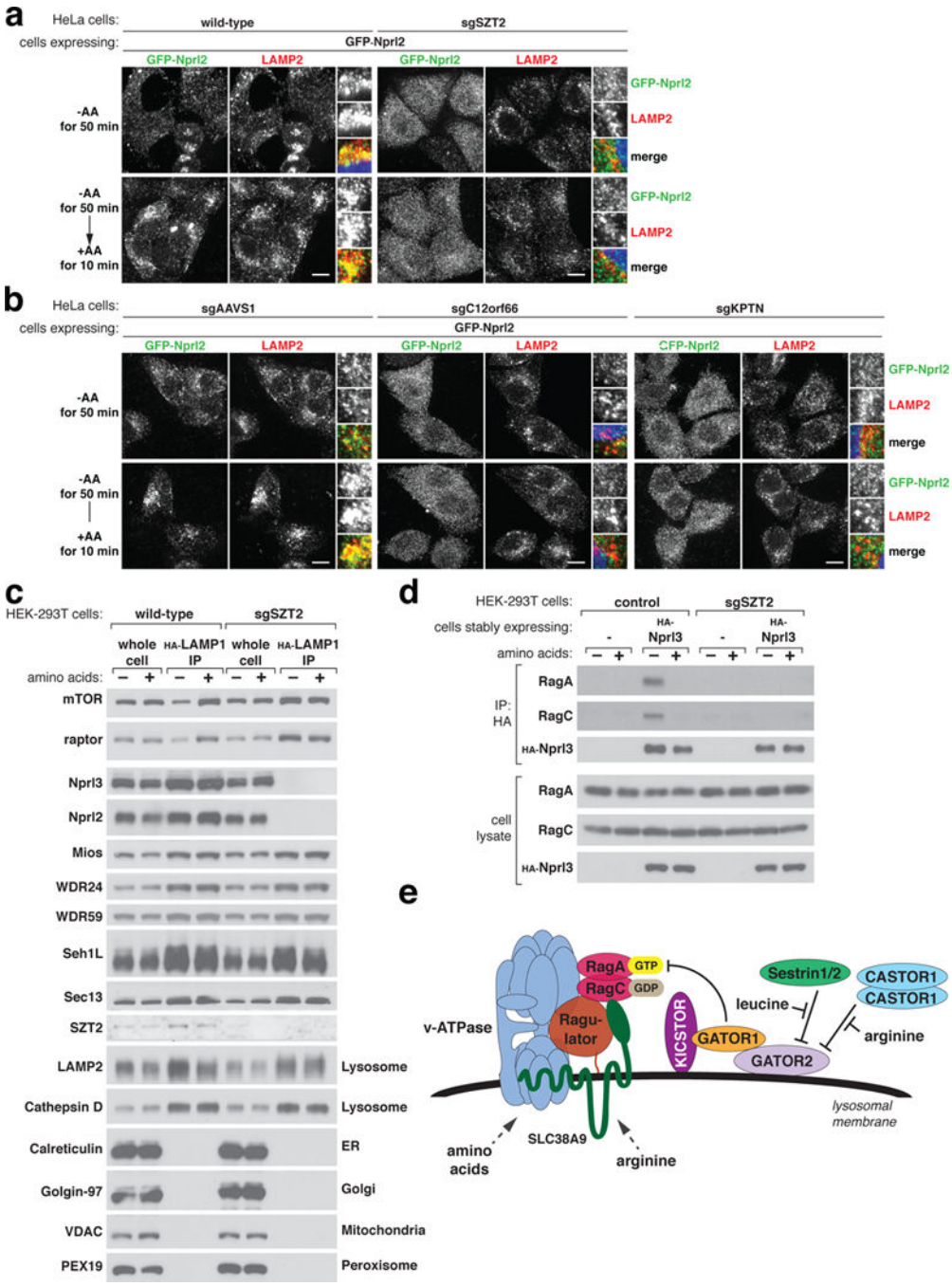



**Figure 4. The lysosomal localization of GATOR1 requires KICSTOR**

a) SZT2 loss renders GATOR1 dispersed throughout the cytoplasm. Nprl2-deficient HeLa cells were reconstituted with GFP-Nprl2 and subsequently modified with the CRISPR/Cas9 system to create SZT2-deficient cells expressing GFP-Nprl2. These cells were starved or starved and restimulated with amino acids for the noted times prior to the detection of GFP and LAMP2 by immunofluorescence. Insets depict selected fields magnified 3.24× and their overlays. Scale bars represent 10 μm.

b) Loss of C12orf66 or KPTN also disrupts the localization of GATOR1 to the lysosome. Cell lines were generated, treated, and analyzed as in (a).

c) In SZT2-deficient cells, GATOR1 is not present on immunopurified lysosomes. Lysosomes were immunopurified from wild-type or SZT2-deficient HEK-293T cells expressing HA-tagged LAMP1 and starved of and stimulated with amino acids as in (a). Lysosomes and whole cell lysates were analyzed by immunoblotting for the levels of the indicated proteins.

d) Loss of SZT2 disrupts the GATOR1-Rag GTPase interaction. Control or SZT2-deficient HEK-293T cells with or without the stable expression of HA-Nprl3 were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Lysates and anti-HA immunoprecipitates were prepared and analyzed by immunoblotting for the levels of the indicated proteins.

e) Proposed role for KICSTOR in the nutrient sensing pathway upstream of mTORC1.